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The *Drosophila* ATM Ortholog, dATM, Mediates the Response to Ionizing Radiation and to Spontaneous DNA Damage during Development

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Summary

Cells of metazoan organisms respond to DNA damage by arresting their cell cycle to repair DNA, or they undergo apoptosis [1]. Two protein kinases, ataxia-telangiectasia mutated (ATM) and ATM and Rad-3 related (ATR), are sensors for DNA damage [2]. In humans, *ATM* is mutated in patients with ataxia-telangiectasia (A-T), resulting in hypersensitivity to ionizing radiation (IR) and increased cancer susceptibility. Cells from A-T patients exhibit chromosome aberrations and excessive spontaneous apoptosis. We used *Drosophila* as a model system to study ATM function. Previous studies suggest that *mei-41* corresponds to ATM in *Drosophila* [3]; however, it appears that *mei-41* is probably the ATR ortholog [4]. Unlike *mei-41* mutants, flies deficient for the true ATM ortholog, dATM, die as pupae or eclose with eye and wing abnormalities. Developing larval discs exhibit substantially increased spontaneous chromosomal telomere fusions and p53-dependent apoptosis. These developmental phenotypes are unique to *dATM*, and both *dATM* and *mei-41* have temporally distinct roles in G2 arrest after IR. Thus, ATM and ATR orthologs are required for different functions in *Drosophila*; the developmental defects resulting from absence of *dATM* suggest an important role in mediating a protective checkpoint against DNA damage arising during normal cell proliferation and differentiation.

Results and Discussion

Phosphoinositide 3-Kinase-Related Kinases Are Conserved in *Drosophila*

The phosphoinositide 3-kinase-related kinases (PIKK) family in mammals includes six subfamilies based on sequence homology and function [5] (Figure 1). Human ataxia-telangiectasia mutated (ATM) and ATM and Rad-3-related (ATR) proteins are the key kinases that transduce signals in response to various types of DNA damage. *Drosophila mei-41* was originally reported as the ATM ortholog [3]. However, sequence analysis reveals that *CG6535*, a gene predicted by the annotated *Drosophila* genome, is more closely related to ATM, and *mei-41* actually belongs to the ATR subfamily (Figure 1) [4]. A related kinase ATX (CG32743 in *Drosophila*) plays a role in eliminating RNA species containing premature termination codons in *C. elegans*, whereas in humans it may have a role in the DNA damage response [6]. Two other PIKK family members *Drosophila* CG2905 and CG5092 are closely related to TRRAP and mTOR, respectively, whereas the catalytic subunit of DNA-dependent protein kinase is not present in *Drosophila*. Among these kinases, flies with mutations in *mei-41* [7] and *mTOR* [8] have been studied in detail, whereas the functions of CG2905, CG6535, and CG32743 in *Drosophila* remain unknown. Here, we describe the function of *dATM* (CG6535) and compare it to that of *Drosophila* ATR (*mei-41*).

dATM Is Required for Viability and Female Fertility

To compare the in vivo functions of *dATM* and *mei-41*, we first examined their developmental expression patterns. The *dATM* gene is expressed at relatively low levels throughout development and at much higher levels in adult females compared to males (Figure 2A). Similarly, *mei-41* is expressed at high levels in adult females and ovaries (data not shown) and was required for meiosis, suggesting a potential role for both genes in the female germ line.

We generated *Drosophila* lines with specific disruptions of the *dATM* gene. A previously generated P element insertion, EP(3)0859, located within the *hsc70-4 (hsc4)* gene, which is 546 bp upstream of *dATM*, disrupts *hsc4* and exhibits homozygous lethality [9] (Figure 2B). In efforts made by others to generate a null allele of the *hsc4* gene, this transposable element was mobilized, resulting in two different imprecise excision deletions of *hsc4* including the *dATM* gene. These are referred to as Δ11 and Δ356 [9] (Figure 2B). The endpoints of the deleted region were confirmed by PCR followed by sequence analysis. *dATM* was predicted to contain 24 exons: Δ11 flies lacked the first 2 exons, whereas the first 8 exons were deleted in Δ356 flies. The flies were extensively out-crossed to wild-type flies to eliminate any additional mutations on the chromosome and then used for further analysis. The lethality resulting from the *hsc4* mutation was rescued transgenetically with a 14 kb genomic fragment encompassing the complete *hsc4* gene, thereby generating "clean" loss-of-function alleles of *dATM* (Figure 2B, Table S1 in the Supplemental Data available with this article online, and [10]).

The majority of flies with the genotype P[hsc4]; Δ11 and P[hsc4]; Δ356 exhibit pupal lethality. However, 12% (Δ11) and 15% (Δ356) of homozygous mutants eclosed and were viable, although they did not survive very long (Table S1). Northern blot analysis of the "escaper" females with the genotype P[hsc4]/Cyo; Δ11, P[hsc4]/Cyo; Δ356, and P[hsc4]/Cyo; EP(3)0859 revealed that the *dATM* transcript was substantially reduced in both Δ11 and Δ356 mutant flies, whereas it was present in EP(3)0859 (Figure 2A). Interestingly, Δ11/Δ356 transheterozygotes exhibited a higher percentage of viability, suggesting that there may be some complementation

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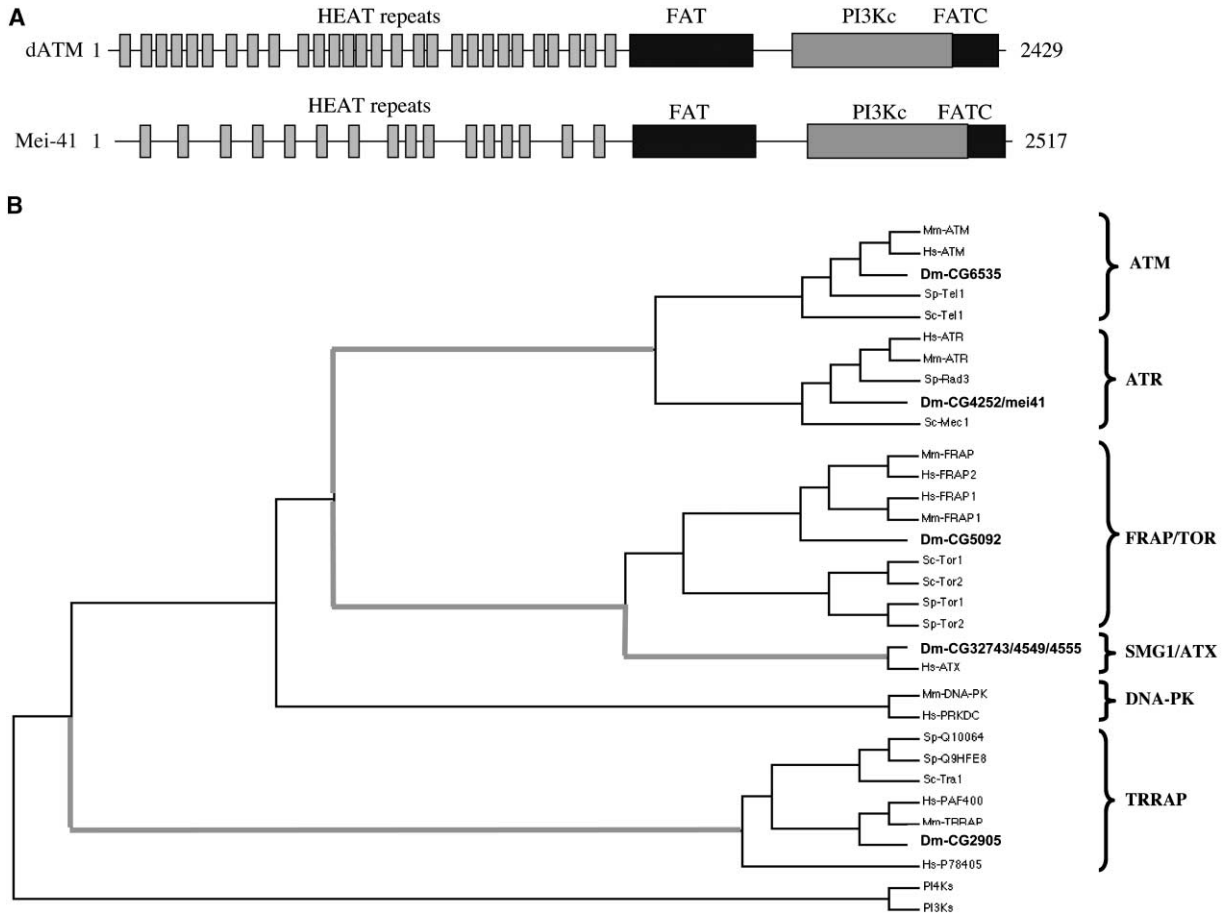

Figure 1. Phylogenetic Tree of the PIKK Family

(A) Schematic representation of the structure of *Drosophila* ATM and mei-41 proteins. The positions of the FAT, PI3Kc, and FATC domains are indicated according to the Simple Modular Architecture Research Tool (SMART). Twenty-eight and sixteen HEAT (Huntington, Elongation factor 3, protein phosphatase 2A, Tor1) repeats of dATM and mei-41, respectively, are shown, as reported previously [24].

(B) PIKK family tree. Sequences of PI3Kc domains for PIKK proteins in selected species (*S.pombe, S. cerevisiae, M. musculus, H. sapiens* and *D. melanogaster*) were obtained from the SPTREMBL (Swiss-Prot TrEMBL) database. The PIKK subfamilies include ATM, ATR, FRAP/mTOR, SMG1/ATX, DNA-PK, and TRRAP.

between the two alleles (Table S1). Moreover, we subsequently obtained two additional *dATM* alleles that exhibit homozygous lethality from the Exelixis transposon disruption collection. Together with similar findings from two accompanying reports indicating that homozygous loss of *dATM* causes lethality, these findings suggest that the Δ11 and Δ356 alleles are strong hypomorphic loss-of-function *dATM* alleles.

dATM, *mei-41*, *grp*, and *dChk2* are close orthologs of mammalian DNA damage checkpoint genes that are all highly expressed in *Drosophila* females relative to males [4]. The encoded checkpoint protein kinases presumably respond to DNA double-strand breaks generated during meiotic recombination. Notably, meiotic recombination in *Drosophila* occurrs only in females. Moreover, mammalian ATM and ATR localize to synapsed and unsynapsed regions of meiotic chromosomes, respectively, suggesting a role for these proteins during meiotic recombination [11]. In *Drosophila*, most *mei-41* alleles are recessive female sterile, and females homozygous for the more fertile hypomorphic *mei-41* alleles exhibit reduced meiotic exchange [12]. The Δ356 *dATM* mutant females did not lay eggs, whereas Δ11 females laid a very small number of eggs that did not hatch, indicating that *dATM* is required for female fertility. In mouse models, ATR is an essential gene [13, 14], whereas ATM is dispensable for normal development and viability, although homozygous mutant mice exhibit immune defects, growth retardation, and sterility [15]. In *Drosophila*, we found the *mei-41*(ATR) mutant to be maternal lethal and nullizygous *dATM* mutants to be lethal. Thus, unlike its mammalian counterpart, *Drosophila* ATM plays a critical developmental role.

dATM Protects Cells from p53-Mediated Apoptosis and Chromosomal Damage during Development

Ataxia-telangiectasia (A-T) patients and ATM-deficient mice exhibit grossly normal development. Interestingly, the *dATM* mutant flies that eclosed all exhibited obvious defects in eye and wing morphogenesis. The eyes were reduced in size and exhibited a uniform surface roughness and missing bristles (Figures 3A and 3C). The wings exhibited notches and, in some cases, blisters (Figures 3D and 3E). When the stronger alleles of *dATM* were crossed to the Δ11 and Δ356 alleles, escapers with

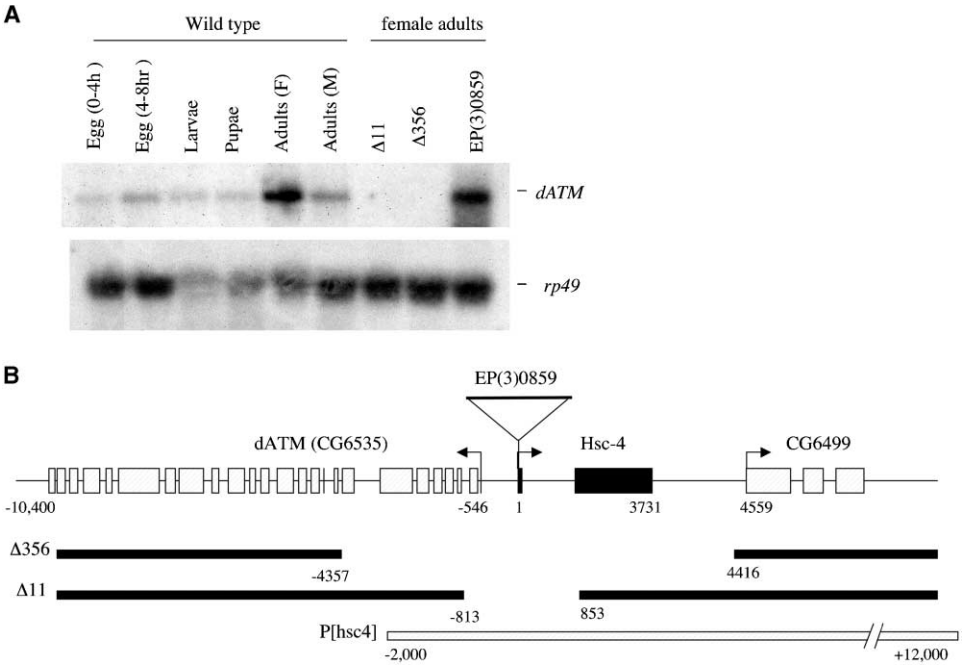

Figure 2. Expression of ATM and *mei-41* in Wild-Type and *dATM* Mutant Flies

(A) Expression of *Drosophila* ATM and *mei-41* in wild-type and *dATM* mutant flies. Total RNA was prepared from various developmental stages of wild-type Oregon R flies and subjected to northern blot analysis. To examine the expression of the *dATM* gene in mutant flies, we used females from the following genotypes for preparing RNA. Δ11: P[hsc4]/CyO;Δ11, Δ356: P[hsc4]/CyO;Δ356, EP(3)0859: P[hsc4]/CyO;EP(3)0859. The same blot was rehybridized to detect ribosomal protein, *rp49*, mRNA for normalization.

(B) Genomic map of the *Drosophila* ATM locus. Hatched boxes indicate the exons from *dATM* and *CG6499*, and dark boxes indicate the exons from *hsc4*. The arrows above the boxes indicate the direction of transcription. The number "1" indicates the start of the *hsc4* transcript as predicted by the Fly Genome Project from the genomic sequence AE003708 [25]. Location of the P element (triangle) is indicated. The genomic region used to generate the transgene P[hsc4] used to rescue the *hsc4* mutation is also indicated.

eye and wing defects were also observed, consistent with the hypomorphic nature of those alleles (Table S1).

To determine the basis for these phenotypes, third-instar wing discs or eye discs were isolated and examined for morphology and for apoptosis by TUNEL staining. Although wing and eye disc morphology appeared to be normal, *dATM* mutant discs were somewhat reduced in overall size (data not shown) and exhibited a substantial increase in TUNEL-positive cells with relatively few apoptotic cells detected within wild-type discs (Figures 3F and 3I). To determine whether the excessive apoptosis is p53-dependent, we introduced a dominant-negative (dn) form of *Dmp53* [16]. The dn-*Dmp53* transgene had previously been shown to block IR-induced apoptosis when expressed in the posterior part of the wing with engrailed-GAL4 [16]. Expression of dn-*Dmp53* in *dATM* mutant wing discs resulted in a substantial decrease in apoptosis (Figures 3J and 3L). Thus, loss of dATM function results in p53-mediated apoptosis during development. In a report by Bi et al. [17], spontaneous apoptosis in *dATM* mutant discs was found to occur even in a p53-zygotic mutant background, suggesting that the observed apoptosis is p53 independent. This apparent discrepancy in findings may reflect the presence of maternal *Dmp53* product in larvae of the homozygous mutant background.

In imaginal eye discs double-stained for TUNEL and the neuronal marker Elav, we observed that the excessive spontaneous apoptosis seen in *dATM* mutants was largely restricted to the proliferating cells anterior to the morphogenetic furrow and that there was evidence of reduced neuronal differentiation and abnormal patterning of differentiated cells (Figure S1). Thus, dATM appears to protect proliferating cells from apoptosis and consequently affects their capacity to differentiate.

In addition to the high level of spontaneous apoptosis observed in *dATM* mutants, we observed that in neuroblasts from dissected brain imaginal discs of *dATM* mutants, a substantial fraction of mitotic cells exhibit evidence of chromosomal damage, particularly, telomere fusions (65.5% of metaphase spreads, $n = 861$). Among metaphase chromosomal spreads, both single and double telomere associations were observed, including fusions between sister chromatids as well as between homologous and nonhomologous chromosomes (Figure 4). Occasionally, several chromosomes were fused together to form a chromosome "chain". Thus, dATM appears to provide a protective function required for maintenance of telomeres and chromosome structure. Similar findings have been made by other groups [17, 18]. Bi et al. report that some of the telomere fusions are dependent on ligase IV and are, therefore, likely to occur by a nonhomolgous end-joining mechanism [17]. These observations are of particular interest, because telomere abnormalities have also been observed in mammalian A-T cells [19]. Moreover, it is likely that the

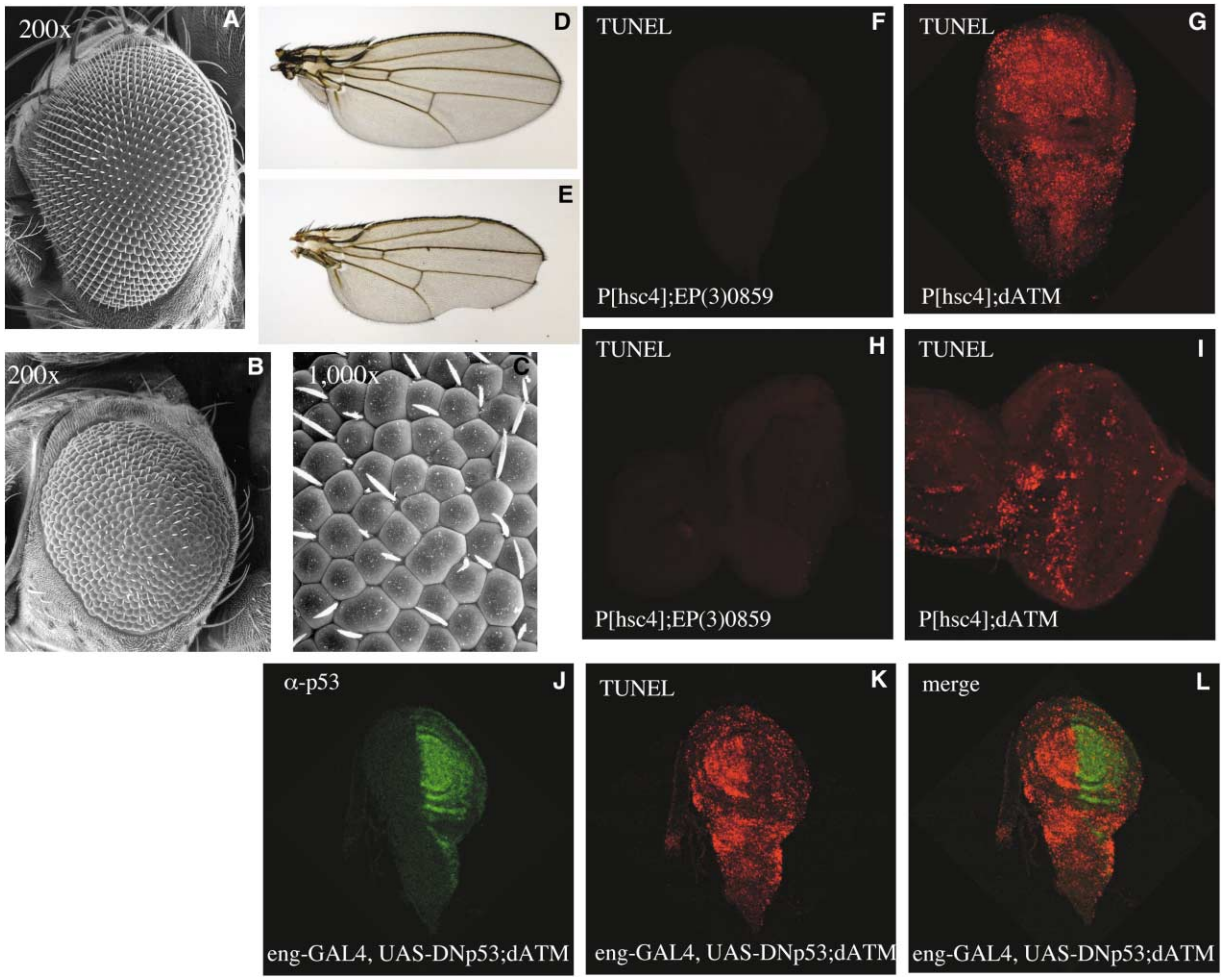

Figure 3. Developmental Defects in *dATM* Mutant Flies

(A and C) *dATM* mutant flies display a rough eye phenotype. Scanning electron micrographs (200× [B] and 1000× [C] magnification) of eyes from viable flies homozygous for a *dATM* mutation ("escapers"), compared to wild-type eyes (A).

(D and E) *dATM* mutant flies exhibit a notched wing phenotype. Adult wings from Oregon R (D) and P[hsc4];Δ356 flies (E) are shown.

(F and I) *dATM* mutant larvae exhibit a substantial increase in spontaneous apoptosis in wing and eye imaginal discs. Third-instar wing (F and G) and eye (H and I) discs from P[hsc4];Δ356 (G, I) and P[hsc4];EP(3)0859 (F,H) were dissected and subjected to TUNEL assays (red) and examined via confocal microscopy.

(J and L) Dominant-negative *Dmp53* suppresses apoptosis in *dATM* mutant flies. Wing discs were dissected from the third-instar larvae with genotype +/p53R155H-pExP-UAS, engrailed-GAL4;Δ356. Discs were stained with anti-*Dmp53* antibody (green, J) and TUNEL (red, [K]). (L) is a merged image of (J) and (K).

presence of dicentric chromosomes, which are unstable, leads to the observed excessive spontaneous apoptosis in *dATM* mutant tissues during development.

DNA Damage Checkpoint Response in *dATM* and *mei-41* Mutants

Like mammalian cells, *Drosophila* cells respond to DNA damage by undergoing cell cycle arrest [4]. In response to IR, cells of wild-type larval discs exhibited delayed entry into mitosis with increased apoptosis. In mammals, the IR-induced G2 arrest is temporally regulated by the different checkpoint kinases, with ATM playing an important role in the initiation of G2 arrest [20] and ATR playing a more prominent role in maintaining the G2 arrest [21]. Moreover, Chk1 and Chk2 have temporally distinct roles in initiating and maintaining G2 arrest, respectively [22].

To examine the temporal roles of ATM and *mei-41* in *Drosophila*, we examined mitosis (as an indicator of cell cycle arrest) at various time points after irradiation. Wild-type wing discs had very few mitotic cells 25 min after irradiation, and no mitotic cells were detected at 1 hr postirradiation (Figure 5). The wing disc cells remained arrested and then reentered the cycle approximately 6 hr later (data not shown). Interestingly, *dATM* mutant wing discs had significantly more mitotic cells 25 min postirradiation as compared to wild-type discs (compare Figure 5E with Figures 5D and S2). At later time points, G2 arrest occurred normally in *dATM* mutant wing discs (Figures 5Gand 5H). In *mei-41* mutant wing discs, cells continued to cycle throughout the time

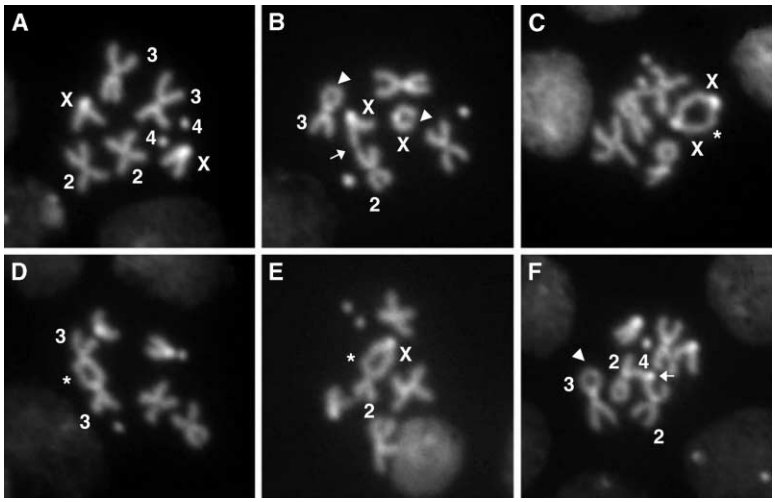

Figure 4. Telomere Fusions in *dATM* Mutant Neuroblasts

(A) Wild-type (Oregon R) metaphase spread showing two X, two second, two third, and two fourth chromosomes. Brains from larvae were treated with colchicine, then squashed and stained with DAPI to visualize mitotic nuclei.

(B–F) P[hsc4]/+;Δ356 *dATM* mutant metaphase spreads showing a variety of telomere fusions. Arrowheads indicate associations between sister chromatids, arrows indicate single telomere associations and asterisks indicate double telomere associations. (B) Associations between sister chromatids of one X chromosome and between sister chromatids of one third (arrowheads), as well as a single telomere association between one X and one second chromosome (arrow). (C) Double telomere association between two X chromosomes (asterisk). (D) Double telomere association between two third chromosomes (asterisk). (E) Double telomere association between one X and one second chromosome (asterisk). (F) Single telomere associations between two second chromosomes and one fourth chromosome (arrow), and an association between sister chromatids of one third chromosome (arrowhead).

points tested as previously reported (Figures 5C, 5F, and 5I and [23]). These results suggest that *d*ATM is involved in the early phase of G2 arrest and *mei-41* has a major role in late response. The observed temporal differences in the IR-mediated checkpoint are consistent with those found in mammals [2].

We also examined potential functional redundancy between *dATM* and *mei-41* by generating double mutants. Flies harboring mutations in both genes exhibited the same excessive level of spontaneous apoptosis seen in the *dATM* mutant larval tissues and no apparent increase in apoptosis following IR (Figure S3); however, we observed that most of the third-instar larvae exhibited black "growths," characteristic of so-called melanotic tumors (Figure S4). The underlying basis for these growths is not known, but it suggests that there is some developmental context in which these two genes function redundantly. The mechanisms underlying the distinct and overlapping functions of *dATM* and *mei-41*/ATR are likely to involve the upstream activation signals

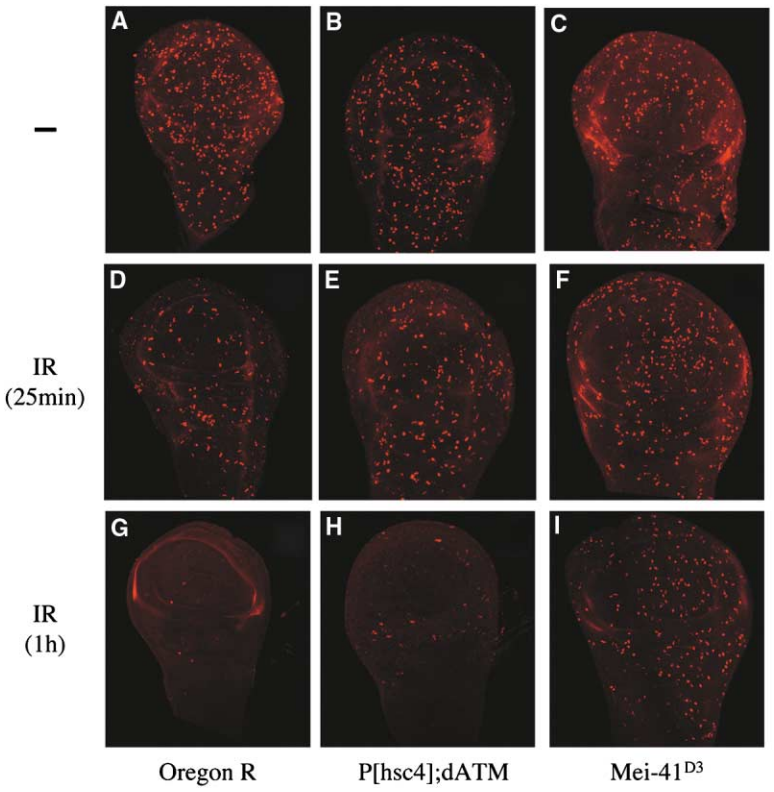

Figure 5. DNA Damage Checkpoint Responses in *dATM* and *mei-41* Mutant Wing Discs

(A–I) *dATM* and *mei-41* have temporally distinct roles in IR-induced G2 arrest. Wild-type (A, D, and G), *dATM* mutant (B, E, and H), or *mei-41* mutant (C, F, and I) third-instar larvae were either untreated (A and C), or treated with 40 Gy IR, and after either 25 min (D and F) or 1 hr (G and I), stained with anti-phospho histone H3 antibody and examined with confocal microscopy to reveal mitotic cells.

and downstream effector substrates for these closely related kinases; as such, the *Drosophila* model provides a genetically tractable system that should prove useful in dissecting their function in an in vivo context.

Conclusions

We examined the function of *Drosophila* ATM and compared its role with that of *mei-41*/ATR during normal development and DNA damage checkpoint responses. Both *dATM* and *mei-41* were highly expressed in female adults and were required for female fertility. Unlike *mei-41*, flies deficient for *dATM* exhibited a substantial increase in spontaneous p53-dependent apoptosis and telomere fusions in developing tissues, leading to lethality or to viable flies with malformed adult tissues. We presume that *dATM* deficiency leads to the accumulation of DNA damage during normal cellular replication and differentiation and that this culminates in p53 activation. Although some ATM functions in mammals are mediated by the Chk2 kinase, the essential developmental role of *dATM* was apparently not mediated by *dChk2*, which is a nonessential gene, indicating that other ATM substrates are required. In addition to their distinct developmental requirements, *dATM* and *mei-41*/ATR perform temporally distinct functions in the DNA damage response to ionizing radiation. Together with the characterization of ATM and ATR functions in mammalian systems, our studies of the *Drosophila* orthologs point to evolutionarily conserved pathways involving two closely related proteins that together regulate genomic integrity during normal development and in response to genotoxic stress.

Supplemental Data

Supplemental Data including Supplemental Experimental Procedures, four additional figures, and a table are available at http://www.current-biology.com/cgi/content/full/14/15/1354/DC1/.

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